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Neutrophils induce proangiogenic T cells with a regulatory phenotype in pregnancy

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Although neutrophils are known to be fundamental in controlling innate immune responses, their role in regulating adaptive immunity is just starting to be appreciated. We report that human neutrophils exposed to pregnancy hormones progesterone and estriol promote the establishment of maternal tolerance through the induction of a population of CD4$^+$ T cells displaying a GARP$^+$CD127^{lo}FOXP3$^+$ phenotype following antigen activation. Neutrophil-induced T (niT) cells produce IL-10, IL-17, and VEGF and promote vessel growth in vitro. Neutrophil depletion during murine pregnancy leads to abnormal development of the fetal-maternal unit and reduced empbryo development, with placental architecture displaying poor trophoblast invasion and spiral artery development in the maternal decidua, accompanied by significantly attenuated niT cell numbers in draining lymph nodes. Using CD45 congenic cells, we show that induction of niT cells and their regulatory function occurs via transfer of apoptotic neutrophil-derived proteins, including forkhead box protein 1 (FOXO1), to T cells. Unlike in women with healthy pregnancies, neutrophils from blood and placental samples of preeclamptic women fail to induce niT cells as a direct consequence of their inability to transfer FOXO1 to T cells. Finally, neutrophil-selective FOXO1 knockdown leads to defective placentation and compromised embryo development, similar to that resulting from neutrophil depletion. These data define a nonredundant function of neutrophil–T cell interactions in the regulation of vascularization at the maternal–fetal interface.

neutrophils | T cells | pregnancy | placenta | regulatory

Establishment of immune tolerance at the fetal–maternal interface has long been associated with the induction of multiple adaptive immunoregulatory mechanisms. For example, the presence of indoleamine 2,3-dioxygenase can dampen maternal T-cell responses to paternal alloantigens, leading to fetal protection (1). Moreover, the programmed cell death ligand (PDL1) is crucial for maternal tolerance in allogeneic pregnancies (2). Finally, the presence of regulatory T cells (Tregs) is instrumental to the maintenance of maternal tolerance (3), where fetus-specific Tregs remain after pregnancy (4) and can expand in subsequent pregnancies (5). Immune tolerance of the fetus relies on the concerted actions of hormones and cytokines, as well as cross-talk between innate and adaptive immune cells (6). Dysregulation of one or more of these components can lead to complications of pregnancy, including fetal growth restriction, preeclampsia, and recurrent miscarriages (7).

Neutrophils, classically considered short-lived cells, provide a first line of defense during infection to help ensure tissue restitution to physiology (8, 9). Emerging evidence indicates that neutrophils also may regulate specific processes typical of adaptive immune responses (10), including antibody production from B cells (11), T-cell suppression (12), and antigen presentation by dendritic cells (13). The ability of neutrophils to affect adaptive immunity appears to be dictated by their activation status (14). Recent studies have suggested the presence of neutrophils in the human decidua that release proangiogenic factors (15, 16); however, their direct influence on pregnancy outcome has not been shown.

In the present study, we describe a role for neutrophils in the establishment of adaptive tolerance during pregnancy. We demonstrate that a population of neutrophils, generated by exposure to pregnancy hormones, is able to induce a unique population of T cells that have regulatory-like and proangiogenic phenotypes. These neutrophil-induced T (niT) cells are necessary for normal placental vascularization and fetal growth during allogeneic pregnancy.

Materials and Methods

Healthy Volunteer and Patient Samples. All volunteers provided informed consent before participating in the study. For in vitro experiments, blood was collected from healthy male volunteers (aged 21–35 y) between 10 and 11 AM. This collection was approved by the East London and The City Local Research Ethics Committee (QMREC2014.61). For patient samples, blood and placental samples from healthy and preeclamptic women were collected at University College, under approval from the London and South-East Research Ethics Committee (13/LO/0287). Patient demographic information is provided in *SI Appendix*, Table S1. Both healthy volunteer and patient blood samples were collected in 3.2% sodium citrate.

Neutrophil Depletion and Reconstitution During Allogeneic Pregnancies. All in vivo experiments were conducted under the Home Office regulation following approval by the Queen Mary University of London Ethics Committee. Here 8- to 12-wk-old Balb/C male mice were mated with aged-matched C57

Significance

Neutrophils are typically known as short-lived cells that act as the first line of defense in response to pathogens. However, emerging data indicate that neutrophils have wider implications in the immune system and have a direct influence on the ensuing immune response. Establishment of successful pregnancy requires immune tolerance at the maternal–fetal interface. Aberrations in normal placental development can lead to complications, including preeclampsia. In this study, we examined a role for maternal neutrophils in maintaining normal pregnancy through their interactions with T cells, resulting in a population of T cells that are both regulatory and proangiogenic and are required for normal placental development. Such interactions are absent in patients with preeclampsia, suggesting a potential therapeutic target for pregnancy-related pathologies.

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BL/6 females. Following identification of a vaginal plug, circulating maternal neutrophils were depleted at days 5 and 8 using a monoclonal neutralizing antibody (Biolegend, clone IA8; 50 μg i.v.). Neutrophils were reconstituted at indicated times during the pregnancy. Neutrophils were made from bone marrow progenitors. Progenitors were isolated via negative selection (Stem Cell Technology). Following an initial lentiviral transduction (24 h) to knock down forkhead box protein 1 (FOXO1), cells were washed and incubated in Iscove's modified Dulbecco's medium supplemented with 20% FCS and 100 ng/mL recombinant murine GCSF for 5 d. Female CD45.1 (Ly5.1) mice were kindly provided by Andrew Cope, King's College London. Mice were originally obtained from Charles River Italy. To check for congenic markers after adoptive transfer of donor CD45.1 neutrophils, cells were stained with Alexa Fluor 488-conjugated anti-mouse CD45.1 (Biolegend; clone A20), along with Alexa Fluor 450-conjugated CD45.2 (eBioscience; clone 104).

Imaging. Neutrophils and autologous T cells were cocultured as described above in Nunc Lab-TekII Chamber Slides (Thermo Fisher Scientific). After coculture, cells were fixed, permeabilized, and then blocked for 3 h with PBS containing 0.1% fish skin gelatin and 1% FCS. Anti-human MRP8 (Abcam; clone CF-145) at 4 °C overnight, followed by staining with secondary antibodies mouse Alexa Fluor 555 and mouse Alexa Fluor 488 along with nuclear stain DAPI (Life Technologies). Human cells were labeled with mouse anti-human FOXO1/FKHR MAb (R&D Systems; clone 597554). Images were acquired using a Zeiss LSM 510 META laser scanning confocal microscope equipped with a 63× Plan-Apochromat objective.

Mouse placentas were embedded in paraffin, and cross-sectional sections were made and imaged to identify all placental layers. Antigen retrieval was carried out using sodium citrate buffer and staining for H&E. Some placental sections were also stained for CD31 (AbD Serotec; clone ER-MP12), cytokeratin-7 (Santa Cruz Biotechnology; FITC-conjugated, clone RCK-105), and DAPI (Life Technologies). H&E images were acquired on a Pannoramic 250 high-throughput scanner (3DHISTECH) at 43× magnification. Immunofluorescence images were acquired on a Leica Ariol System at 20× magnification. Image analysis was performed using ImageJ, and scale bars were added onto the overlaid images.

Statistics. Data were analyzed by one- or two-way ANOVA, followed by Bonferroni's post hoc test using GraphPad Prism 5. Alternatively, several experiments were analyzed by Student's *t* test. Data are presented as mean ± SEM, and a P value < 0.05 was considered to indicate statistical significance.

Results

Human Neutrophils Exposed to Pregnancy Hormones Induce T Cells with a Regulatory-Like Phenotype and Proangiogeneic Activity. To study a potential role for neutrophils in maternal tolerance, we tested whether neutrophils exposed to pregnancy hormones could affect T-cell responses. Neutrophils from healthy male donors were used to minimize any effects from endogeneous progesterone and estrogen. Male cells express the same levels of estrogen and progesterone receptors on leukocytes as females (17), and thus respond to both hormones. Neutrophils (*SI Appendix*, Fig. S1*A*) were incubated with either progesterone (P; *SI Appendix*, Fig. S1*B*) or the placental estrogen estriol (E3), or in combination (hereinafter E3P), at 100 ng/mL, physiological pregnancy levels. Treatment of neutrophils for 30 min with E3P led to a distinct $CD16^{lo}CD62L^{lo}CD11b^{lo}AnxA1^{hi}$ phenotype (Fig. 1*A*), typical of an anti-inflammatory/quiescent neutrophil status (14, 18). The addition of E3P neutrophils to autologous carboxyfluorescein succinimidyl ester (CFSE)-labeled T cells (+ anti-CD3, anti-CD28 mAbs), enhanced T-cell proliferation (Fig. 1*B*). These T cells produced significant levels of IL-10, IL-17, IL-2, and IL-5, but not of IFN-γ, compared with T cells activated in the presence of vehicle-treated neutrophils or on their own (Fig. 1*C* and *SI Appendix*, Fig. S1*C*). Neutrophils cultured alone did produce cytokines, confirming their purity and the absence of contaminating monocytes (*SI Appendix*, Fig. S1*C*).

Given the increased levels of regulatory cytokines detected in E3P-treated neutrophil–T-cell cocultures, we defined their phenotypic and functional characteristics. A significant increase in the proportion of total $CD4^+CD45RO^+$ T cells expressing high levels of FOXP3 was quantified in the divided T-cell population, with low levels of CD127 and high levels of the latency-associated peptide receptor Glycoprotein A Repetitions Predominant (GARP) (Fig. 1*D* and *SI Appendix*, Figs. S1*D* and S2*A*). The induction of Tregs by E3P-treated neutrophils relied on TCR stimulation, given that neutrophils failed to induce FOXP3 expression in unstimulated T cells (*SI Appendix*, Fig. S2*B*). Coculture with neutrophils led to equal expansion of $CD4^+CD45RO^+$ T cells from the starting naïve T-cell population, regardless of neutrophil hormone treatment (Fig. 1*E*, *Left*); however, expression levels of FOXP3 and GARP were induced in $CD45RO^+$ T-cells selectively when cocultured with E3P neutrophils (Fig. 1*F*, *Right*), indicating that this pathway of differentiation occurs during T-cell priming.

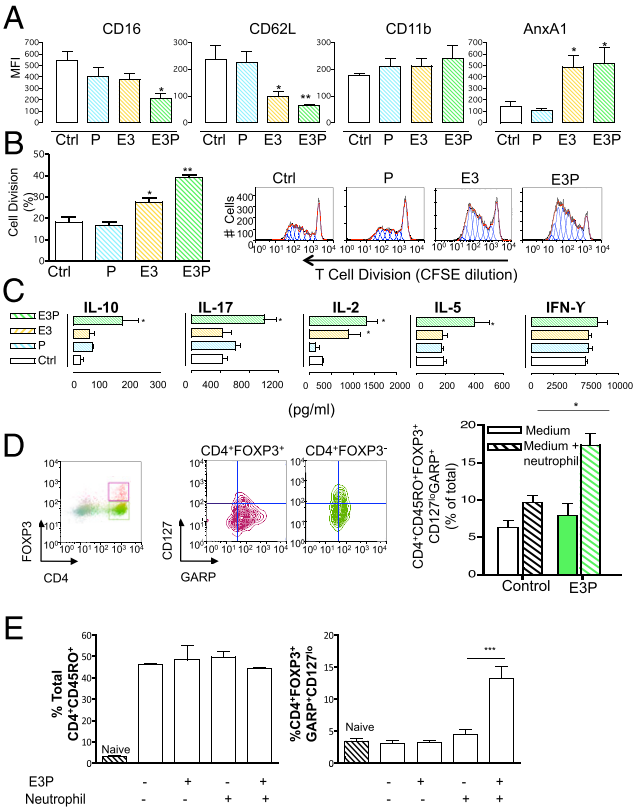

Fig. 1. Human neutrophils treated with estriol and progesterone display a distinct phenotype and induce Tregs from a naïve T cell population. (*A*) Neutrophils from healthy male donors were treated with progesterone (P; 100 ng/mL) or estriol (E3; 100 ng/mL) or in combination (E3P) for 30 min and then washed. Neutrophil phenotype was analyzed by flow cytometry for surface markers CD16 (FcγRIII), CD62L, CD11b, and the neutrophil anti-inflammatory protein AnxA1. *$P < 0.05$, **$P < 0.01$ compared with control-treated neutrophils. (*B*) Neutrophils were treated with hormones as described in *A* and then cocultured with autologous lymphocytes (labeled with 3 μM CFSE) at a 1:1 ratio for 5 d in the presence of 2 μg/mL soluble anti-CD3 and anti-CD28 antibodies. The scheme of cell treatment is reported in *SI Appendix*, Fig. S1*B*. *$P < 0.05$ compared with control. (*C*) Supernatants were collected from cocultures and from lymphocytes or neutrophils cultured on their own, in the presence or absence of P and E3 (100 ng/mL each). Cytokine levels were quantified using a multiplex assay. *$P < 0.05$ compared with control treatments. (*D*) After culture, lymphocytes or lymphocytes that had been cocultured with neutrophils (with or without P and E3) were stained for CD4, CD45RO, and FOXP3, as well as for GARP and CD127. *$P < 0.05$ compared with medium. (*E*) CD45RA-naïve T cells were isolated by negative selection from healthy male donors and cocultured at a 2:1 ratio with control- or E3P-treated neutrophils for 5 d (plus 2 μg/mL soluble anti-CD3 and anti-CD28 antibodies).

Further analyses revealed that these neutrophil-induced T (niT) cells coexpressed GARP and PD1 (*SI Appendix*, Fig. S2*C*), a phenotype typical of functionally suppressive human TCR-activated FOXP3$^+$ Tregs (19). Suppression assays revealed that niT cells were also functionally suppressive, which was cell contact-dependent (*SI Appendix*, Fig. S2 *D* and *E*). This finding suggested signaling of neutrophils to T cells during activation, either by direct cell–cell contact or through the release of large cell material (>0.4 μm). The hormone effect on neutrophils was specific to E3P; treatment of neutrophils with third trimester levels of estrone, 17-β-estradiol, or β-human chorionic gonadotrophin (in the presence or absence of 100 ng/mL progesterone) did not induce niT cells following coculture (*SI Appendix*, Fig. S3*A*). Neutrophils from healthy female donors (regardless of stage of menstrual cycle) induced niT cells even without stimulation with E3P (*SI Appendix*, Fig. S3 *B* and *C*), likely owing to continued exposure to high levels of progesterone, given that this hormone on its own was sufficient to induce niT cells (*SI Appendix*, Fig. S1*D*).

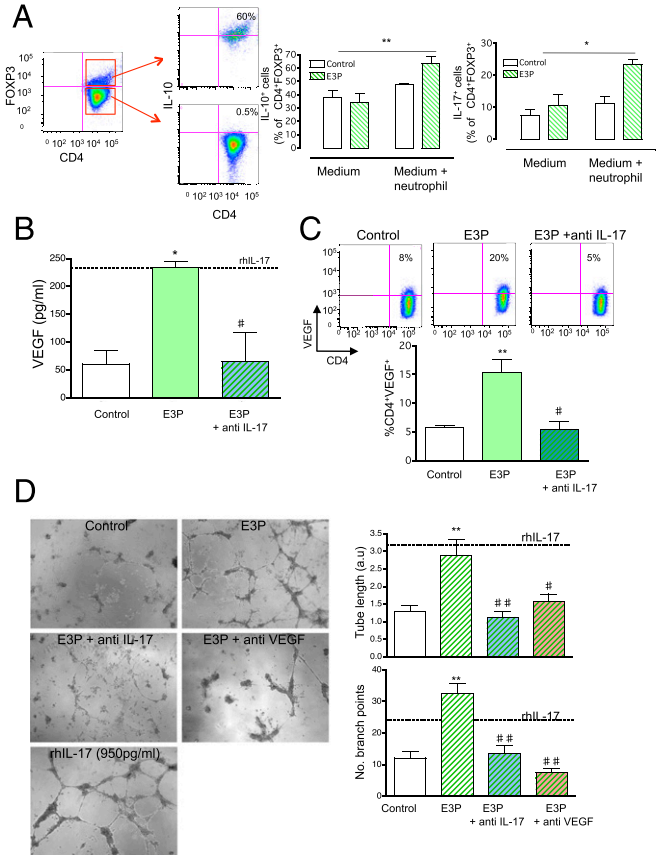

Fig. 2. Human neutrophil-induced T cells secrete regulatory cytokines and are proangiogeneic. (*A*) Control (white bars) or E3P (green-lined bars) CD4$^+$ lymphocytes cocultured with neutrophils were stained for intracellular FOXP3 and IL10 (*Left*; gated on CD4$^+$FOXP3$^+$; $^{**}P < 0.01$ compared with medium) or IL17 (*Right*; $^*P < 0.01$). $^{**}P < 0.01$. (*B*) ELISA for VEGF carried out with the supernatants of cocultures described in Fig. 1*A*. In some cases, IL-17 was blocked during coculture at a concentration of 630 ng/mL, which was the optimal dose to inhibit the level of IL-17 released in these cocultures (950 pg/mL). As a positive control, recombinant human IL-17 was added to medium at the same concentration as in the cocultures. (*C*) Intracellular staining for VEGF in CD4$^+$ T cells following the cocultures described above. (*D*) Vessel growth assay using growth factor-reduced Matrigel. $^*P < 0.05$ compared with control; $^{\#}P < 0.05$ compared with E3P. In all cases, data are mean ± SEM of three to five experiments, with three to five donors per experiment.

Further analyses revealed that niT cells produce IL-10 and IL-17 (Fig. 2*A*). IL-17–producing Tregs have been described previously (20). IL-17 has been shown to have proangiogenic functions by inducing the release of growth factors, including VEGF (21). Supernatants from T cells cocultured with E3P neutrophils also contained significant levels of VEGF. To confirm that the VEGF production in our system was induced by IL-17, we used an anti–IL-17 neutralizing strategy, which impaired VEGF production (Fig. 2*B*). More specifically, intracellular staining confirmed VEGF production by niT cells (Fig. 2*B*). We analyzed the proangiogenic function of niT cells in vitro using Matrigel-based assays. Fig. 2*D* shows that supernatants from niT cells (E3P), but not controls, significantly augmented both vessel length and number of branch points, and was inhibited on IL-17 or VEGF neutralization.

Depletion of Neutrophils During Pregnancy Leads to Impaired Embryo and Placenta Development. Although activated neutrophils have been implicated in the pathophysiology of preeclampsia (22), very little is known about the physiological role of quiescent neutrophils in pregnancy, despite their increased circulating numbers during gestation (23). Our observations in the human system in vitro indicate a potential protective role for quiescent neutrophils in pregnancy. Therefore, we sought to validate this hypothesis in murine pregnancy. Pregnant C57BL/6 females previously mated with BALB/c males were treated with anti-Ly6G monoclonal antibody (clone IA8, 50 μg i.v.; *SI Appendix*, Fig. S3) to deplete maternal neutrophils. Depletions were performed on days 5 and 8 of pregnancy, a time of active placental development in the mouse (24).

Analysis of pregnant mice at day 12 revealed systemically fewer Ly6G$^+$ neutrophils in neutrophil-depleted mice compared with isotype control-treated mice. Importantly, neutrophil depletion led to a significant reduction in the numbers of CD4$^+$FOXP3$^+$ Tregs retrieved in the draining lymph nodes (Fig. 3*A* and *SI Appendix*, Figs. S4 and S5). The physiological role of niT cells in pregnancy was confirmed by our finding of significantly smaller fetuses (Fig. 3 *B* and *C*) and placentas (Fig. 3 *C* and *D*) of neutrophil-depleted pregnancies compared with control pregnancies, a phenotype typical of preeclampsia in humans (25).

Histological analyses of placentas in neutrophil-depleted mice revealed a disorganized structure with reduced thickness of the maternal decidua, which contains the maternal spiral arteries (decidua basalis; Db) (Fig. 3*E*), coupled with poor trophoblast invasion into the maternal layer (Fig. 3*F*). Both fetal and placental size are determined by maternal uteroplacental perfusion of the placenta, which requires coordinated trophoblast invasion and recruitment and remodeling of the maternal spiral arteries (26). Given that IL-17 can promote trophoblast invasion in vitro (27), it is conceivable that niT cell-derived IL-17 might play a role in this event. Loss of peripheral (induced) Tregs is known to hinder placental development (28). Collectively, this evidence suggests that maternal neutrophils are required for efficient uteroplacental circulation.

Transfer of Neutrophil-Derived FOXO1 Is Required for niT Cell Induction. Neutrophils can regulate specific host responses through the release of microstructures, including neutrophil extracellular traps (NETS) (29), apoptotic bodies, and microvesicles, which contain a variety of immune-modulatory proteins (30). Exposure to E3P induced a proapoptotic neutrophil phenotype with low surface expression of CD16 (31) (Fig. 1*A*). Conversely, the cytokine GM-CSF, which promotes leukocyte survival (32), attenuated the proportion of late apoptotic neutrophils (annexin-V$^+$, PI$^+$) by 30% and 50% in control and E3P-treated neutrophils, respectively (Fig. 4*A*). Time-course analyses indicated that neutrophil apoptosis following exposure to E3P begins at 24 h of coculture (*SI Appendix*, Fig. S6*A*).

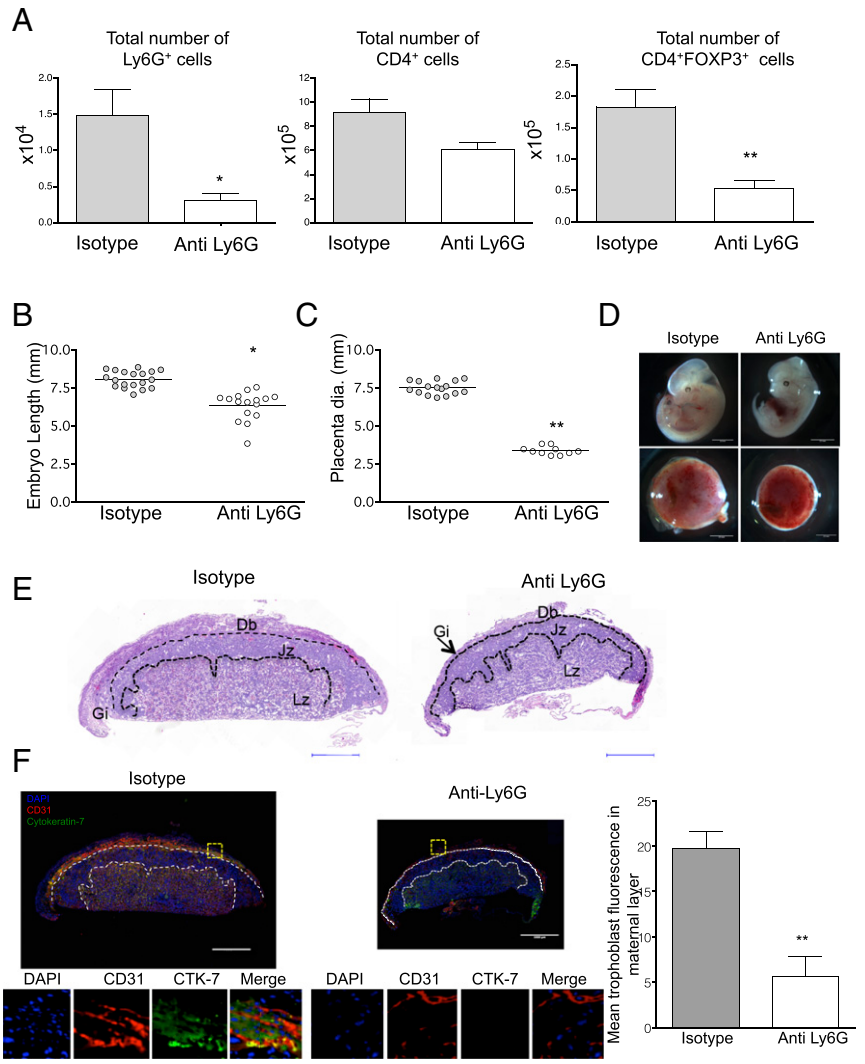

Fig. 3. Neutrophil depletion during allogeneic pregnancy leads to abnormal placentation. Balb/C males were mated with C57 BL/6 females. After identification of vaginal plugs, circulating maternal neutrophils were depleted using a monoclonal neutralizing antibody, or some pregnant females were treated with isotype control antibody (both antibodies at 50 μg i.v.), at days 5 and 8, and pregnant animals were killed at day 12. (*A*) Absolute numbers of Ly6G$^+$ neutrophils, CD4$^+$ T cells, and CD4$^+$FOXP3$^+$ Tregs in the draining para-aortic lymph nodes. *$P < 0.05$; **$P < 0.01$ compared with isotype control-treated animals. (*B*) Crown-to-rump measurements obtained to determine embryo lengths. (*C*) Differences in placenta diameter from isotype control and neutrophil-depleted mice. *$P < 0.05$; **$P < 0.01$ compared with isotype control-treated animals. (*D*) Representative images of embryos and placentas from isotype control and neutrophil-depleted animals. (Scale bar: 2.5 mm.) (*E*) H&E staining of placentas from two groups of mice to examine the organization of placental layers (dotted lines). Gi, giant cell trophoblasts; Db, decidua basalis; Jz, junctional zone; Lz, labyrinthine zone. (Scale bar: 1,000 μm.) (*F*) Immunofluorescent staining of placentas for CD31 (red), trophoblasts (cytokeratin-7; green), and cell nuclei with DAPI (blue). Dotted lines indicate placental layers as described in *E*. Yellow dotted square indicates zoomed-in section of the Db layer to more closely examine trophoblast invasion. Invasion of trophoblasts into the maternal layer was quantified using ImageJ software. **$P < 0.01$ compared with isotype control. In all cases, data are mean ± SEM of two to three experiments with three to four animals per group.

To gain insight into the mechanism of niT-cell induction, we investigated the ability of E3P- and/or GM-CSF–treated neutrophils to transfer cytoplasmic material [monitored through myeloid-related protein 8 (MRP8) staining] to T cells. Whereas treatment of neutrophils with E3P significantly promoted the transfer of neutrophil proteins to T cells, leading to the generation of niT cells, this response was reduced and/or inhibited when neutrophils were treated with GM-CSF (Fig. 4 *B* and *C*). Finally, induction of FOXP3 expression was specific to neutrophil-derived apoptotic bodies, as demonstrated by the ineffective coculture of T cells with monocytic apoptotic bodies (*SI Appendix*, Fig. S6*B*).

We next sought to identify the molecular interactions mediating niT-cell induction. We first interrogated the role of the neutrophil-abundant protein Annexin-A1 (AnxA1), because its up-regulation is a feature of an anti-inflammatory neutrophil phenotype as promoted by female sex hormones (14, 18). Pretreatment of freshly isolated human neutrophils with recombinant AnxA1, at an optimal concentration of 3 nM, reproduced E3P exposure and subsequent niT-cell induction; however, direct addition of AnxA1 to T-cells failed to do so (Fig. 5*A*).

These observations were confirmed by experiments with neutrophils and T cells from AnxA1 KO (33) and WT mice. Neutrophils from AnxA1 KO or WT mice were treated with E3P and cocultured as described above. E3P-treated neutrophils from WT animals induced FOXP3 expression equally in WT T cells and AnxA1 KO T cells. In contrast, neutrophils from AnxA1 KO mice failed to induce FOXP3 expression in T cells irrespective

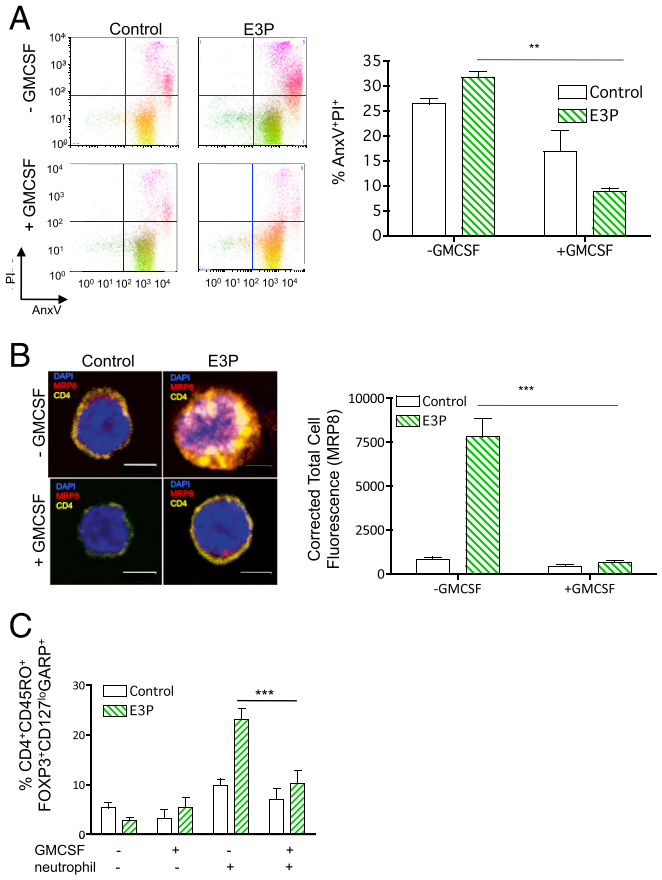

Fig. 4. Human neutrophils induce Tregs via apoptotic bodies. (*A*) Control- and E3P-treated (Fig. 1) neutrophils were incubated with or without GMCSF (50 ng/mL) in coculture with autologous T cells. After a 24-h coculture, cells were stained with annexin-V and PI. Based on forward/side scatter, neutrophils were gated, and the degree of apoptosis (**$P < 0.01$) was compared with control. (*B*) T cells were stained for CD4 (yellow) MRP8 (red), and DAPI (blue) and analyzed by confocal microscopy. ***$P < 0.001$ compared with without GMCSF. (Scale bar: 7 μm.) (*C*) Flow cytometry analyses of niT cell induction in the presence or absence of GMCSF with respect to CD45RO, FOXP3, CD127, and GARP. ***$P < 0.001$. In all cases, data are mean ± SEM of four to five experiments with more than three distinct donors per experiment.

of hormone treatment (Fig. 5*B* and *SI Appendix*, Fig. S7*A*). Taken together, these data indicate that AnxA1 does not directly induce differentiation of niT cells, but it may facilitate the transfer of neutrophil-derived signaling proteins necessary for niT-cell formation.

The transcription factor FOXO1 is the master regulator necessary for FOXP3 expression in inducible Tregs (34–36). Intriguingly, FOXO1 is expressed by neutrophils, where it is endowed with proapoptotic functions (37). Both estrogen and progesterone can up-regulate FOXO1 in the placenta during pregnancy, and this transcription factor is important for normal placental development and decidualization (38, 39). Therefore, we tested whether E3P exposure up-regulates FOXO1 expression in neutrophils, which on apoptosis could then transfer the transcription factor via apoptotic bodies to induce FOXP3 expression in the targeted T cells. Confocal microscopy revealed an ∼30-fold increase in FOXO1 expression in neutrophils treated with E3P compared with untreated cells (Fig. 5*C*). Subsequently, EP3 neutrophils were labeled with anti-FOXO1 antibody by brief osmotic shock and kept overnight in serum-free media to generate neutrophil apoptotic bodies. Feeding these apoptotic bodies to autologous T cells effectively transferred substantial amounts of neutrophil-derived FOXO1 to T cells (Fig. 5*D*) and FOXP3 expression (Fig. 5*E*), whereas this transfer did not occur with apoptotic bodies from control neutrophils. Consistent with a causal link between FOXO1 transfer via apoptosis and niT-cell induction, pharmacologic inhibition of FOXO1 significantly inhibited their ability to undergo apoptosis (*SI Appendix*, Fig. S7*B*) and failed to induce Tregs (Fig. 5*F*). Neutralization of AnxA1 on E3P-derived neutrophil apoptotic bodies significantly attenuated the transfer of FOXO1 via neutrophil apoptotic bodies on induction of FOXP3 (Fig. 5*G*; compare with 5*D*).

Activated Neutrophils from Preeclamptic Pregnancies Fail to Induce niT Cells. Many of the pathological features that we observed in our in vivo model are compatible with those previously reported in preeclamptic pregnancies, including loss of neutrophil quiescence (22) and reduction of circulating Tregs (40). Preeclampsia is a multiorgan syndrome of pregnancy that most commonly affects first-time pregnancies in the second trimester of pregnancy (2–7%) and it is a leading cause of maternal and fetal morbidity and mortality (41). Lower levels of estriol have been associated with this pathology (42).

Although the vascular alterations occurring in pre-eclampsia are well established (43), the impact of immune components in its pathogenesis is less clear (7). Thus, we next interrogated the role of neutrophils in niT-cell induction in women with preeclampsia. We observed a significant attenuation in plasma levels of both progesterone and estriol in preeclamptic women compared with healthy pregnancies (*SI Appendix*, Fig. S8). We next compared neutrophil phenotypes in healthy and preeclamptic patients (*SI Appendix*, Table S1). Elevated CD11b expression in neutrophil from preeclamptic patients has been reported previously (22). Our present analysis demonstrated that blood and placental neutrophils from preeclamptic patients displayed an activated phenotype, with high levels of CD16, CD62L, and CD11b but lower levels of AnxA1 (Fig. 6*A* and *SI Appendix*, Fig. S9 *A* and *B*). In this context, we note how defective AnxA1 signaling has been associated with preeclampsia, because it may result from autoantibodies to the protein or from reduced placental expression of its receptor FPR2/ALX (44, 45). To make a direct comparison with the observed attenuation in fetal size following our in vivo neutrophil depletion model during pregnancy (Fig. 3), we could quantify that offspring born to our preeclamptic group had a significantly lower median birth weight percentile compared with offspring born to mothers in our healthy group [preeclampsia: 22nd percentile (range, 11th–35th percentile) vs. healthy: 52nd percentile; range, 44th–68th percentile], suggesting fetal growth restriction in our preeclamptic population, akin to that seen in our mouse model.

We also found that a lower proportion over 24 h of neutrophils from preeclamptic patients entered apoptosis (∼10% vs. 20% of healthy) (Fig. 6*C*), suggesting attenuated apoptotic body formation. In line with this hypothesis and congruent with the data in human neutrophils and T cells, we found a significant reduction in the transfer of neutrophil-derived MRP8 and AnxA1 in preeclamptic patient-derived T cell/neutrophil cocultures compared with cocultures of cells from healthy pregnancies (Fig. 6*C*). This finding was also associated with attenuated FOXO1 transfer to T cells from neutrophils from preeclamptic patients compared with their healthy counterparts (Fig. 6*D*).

In line with previous reports (40, 46), we observed a significant reduction in circulating and placental $CD4^+FOXP3^+$ Tregs in our preeclamptic patients (*SI Appendix*, Fig. S9 *C* and *D*). Therefore, we compared the ability of neutrophils isolated from preeclamptic and healthy donors to induce niT cells. Coculture of freshly isolated neutrophils and T cells from healthy samples (blood and placenta) led to the induction of functionally suppressive niT cells (*SI Appendix*, Fig. S10 *A and B*), which produced both IL-10 and IL-17 (*SI Appendix*, Fig. S10 *D* and *E*). In

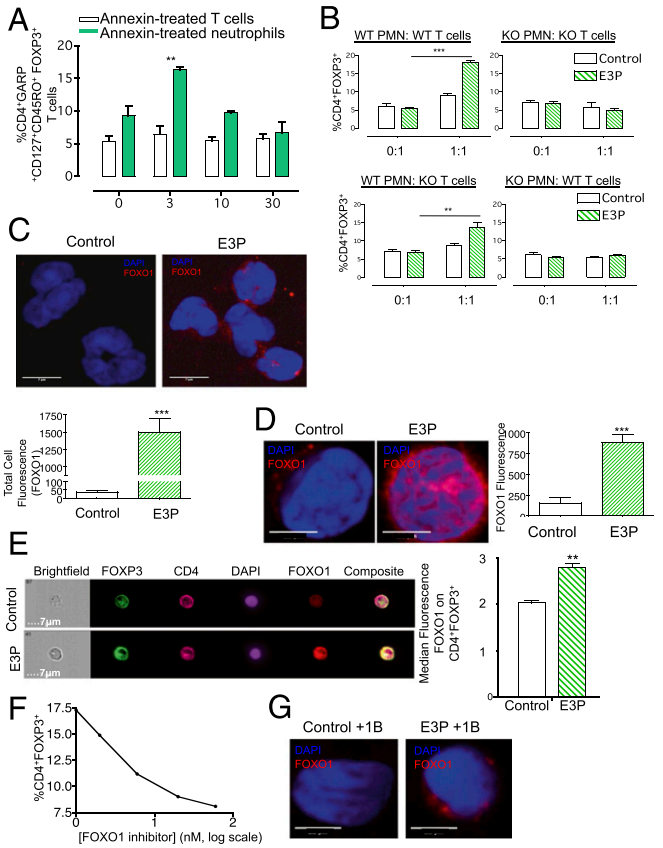

Fig. 5. Neutrophils induce FOXP3 to T cells via the transfer of FOXO1. (*A*) The effect of recombinant human AnxA1 on niT cell induction was assessed by treating either T cells alone (white bars) or in coculture following direct treatment of neutrophils (green bar). $^{**}P < 0.01$ compared with neutrophil coculture in the absence of AnxA1 treatment. (*B*) Neutrophils and splenic T cells were isolated from male WT and AnxA1 KO mice. Neutrophils were treated with E3P (green lined bars) or left untreated (white bars) and then cocultured (at a 1:1 ratio) for 3 d with autologous or counterpart (WT or KO) T cells, stimulated with 2 μg/mL anti-CD3 and anti-CD28 antibodies. T cells were stained for CD4 and FOXP3. $^{***}P < 0.001$ compared with T cells in the absence of neutrophils. (*C*) Neutrophils from male donors were treated with progesterone and estriol (E3P; 100 ng/mL each) for 30 min, washed, and stained with DAPI and mouse anti-human FOXO1 (10 μg/mL) in 0.1% Triton X-100 to gently permeablize the cells, followed by the addition of secondary Alexa Fluor 555 antibody. FOXO1 expression by neutrophils was quantified by confocal microscopy. (Scale bar: 7 μM.) $^{**}P < 0.001$ compared with control. (*D*) FOXO1 protein contained by E3P neutrophils was labeled with a fluorochrome-conjugated antibody as in *B*. After an 18-h incubation, FOXO1-labeled apoptotic bodies were collected and cocultured with autologous T cells for 3 d in the presence of 2 μg/mL soluble anti-CD3 and anti-CD28 antibodies. FOXO1 transfer to T cells was quantified by confocal microscopy and measured as corrected total cell fluorescence (CTCF). (Scale bar: 3.5 μM.) $^{***}P < 0.001$ compared with control. (*E*) T cells, cultured as described in *D*, were stained for CD4 (pink), FOXP3 (green), and FOXO1 (red) expression and then counterstained with DAPI (purple). The intensity of FOXP3 and FOXO1 expression in the same cell was determined using ImageStreamX analysis by comparing bright detail similarity (median fluorescence) of the two. $^{**}P < 0.01$ compared with control. (Scale bar: 7 μm.) (*F*) The ability of E3P-treated neutrophils to induce Tregs was determined following neutrophil-specific inhibition of FOXO1. Log scale is shown. (*G*) T cells were cocultured with neutrophil apoptotic bodies as described in *D*. In addition, AnxA1 was neutralized on neutrophils before apoptotic body formation, and the ability of neutrophil-derived FOXO1 transfer to T cells was measured by confocal microscopy. Data are mean ± SEM of two experiments conducted with three donors per experiment.

contrast, neutrophils from preeclamptic patients failed to induce niT cells, and the minute proportion of these cells found in the cocultures displayed low levels of IL-10 and IL-17 (Fig. 6*E*). Importantly, treatment of neutrophils from preeclamptic patients with E3P rectified their defect and led to the induction of niT cells and ensuing production of IL-10 and IL-17 (Fig. 6*E* and *SI Appendix*, Fig. S9 *D* and *E*). Production of IL-17 by niT cells may deliver important functions, including microbial defense and promotion of angiogenesis (21), both of which are vital during pregnancy.

FOXO1-Deficient Neutrophils Fail to Induce niT Cells and to Restore Embryo and Placental Development. To validate the mechanistic importance of neutrophil-derived FOXO1 transfer to T cells, we investigated whether neutrophil-derived protein could be transferred to T cells in vivo, and whether loss of neutrophil-derived FOXO1 was responsible for the pregnancy defects observed following neutrophil depletion. We tested the former question by injecting CFSE-labeled neutrophils at day 6 of pregnancy, following an initial neutrophil depletion at day 5. Spleen, uterine-draining para-aortic lymph nodes, and nondraining brachial lymph nodes were harvested 48 h later. As shown in *SI Appendix*, Fig. S11*A*, T cells from draining lymph nodes expressed high levels of CFSE, whereas spleen and nondraining lymph node T cells did not. More specifically, a large proportion of $CD4^+FOXP3^+$ T cells expressed $Ly6G^+$ $CFSE^+$ (*SI Appendix*, Fig. S11*B*), suggesting transfer of neutrophil-derived products to T cells in vivo.

To validate the relevance of the FOXO1/FOXP3 axis to niT-cell generation in pregnancy, we selectively knocked down FOXO1 in murine neutrophils using lentiviral delivery. Two different short-hairpin RNAs (shRNAs) specific for FOXO1 (FOXO1 30 and FOXO1 42) and a nonspecific control were transduced into bone marrow progenitor cells (*SI Appendix*, Fig. S12 *A* and *B*). After 24 h, progenitor cells were stimulated with GCSF for 5 d to develop into $Ly6G^+$ neutrophils (~80% after puromycin treatment; *SI Appendix*, Fig. S12*C*). In vitro, FOXO1-sufficient neutrophils were capable of inducing niT cells when cocultured with naïve T cells, whereas FOXO1-deficient neutrophils were ineffective (*SI Appendix*, Fig. S13*A*). This correlated with a reduced ability to induce IL-10, IL-17, and VEGF (*SI Appendix*, Fig. S13 *B–D*).

In a final series of experiments, we wished to establish the physiological relevance of this mechanism in vivo. To this end, we reconstituted neutrophil-depleted pregnant mice with either FOXO1-sufficient (nonspecific) or FOXO1-deficient (FOXO1 30 or FOXO1 42) neutrophils (3×10^6 i.v.) at days 6 and 9 of pregnancy. Neutrophil depletion was performed at days 5 and 8 as described above. Pregnant mice were analyzed at day 12 of gestation. Similar numbers of $Ly6G^+$ neutrophils were present in the uterine-draining lymph nodes following reconstitution with either FOXO1-sufficient or FOXO1 knockdown neutrophils (Fig. 7*A*, *Left*). Similarly, there was no difference in the number of $CD4^+$ T cells (Fig. 7*A*, *Middle*). In contrast, the number of $CD4^+FOXP3^+$ T cells was significantly decreased (~75%) following reconstitution with FOXO1-deficient neutrophils compared with FOXO1-sufficient neutrophils (Fig. 7*A*, *Right*). This was accompanied by a diminished proportion of cells making IL-10, IL-17, and VEGF following reconstitution of FOXO1 knockdown neutrophils.

To conclusively establish the effects of reconstituted donor neutrophils on Treg induction and to confirm the results shown in *SI Appendix*, Fig. S11, we used neutrophils from CD45.1 congenic mice. The purity of injected donor $CD45.1^+$ neutrophils is shown in *SI Appendix*, Fig. 13*E*. Bone marrow progenitors from these mice were transduced, cultured into neutrophils, and finally injected into pregnant CD45.2 recipients. We evaluated the presence of donor $CD45.1^+$ neutrophils at two distinct time points after injection: 16 h and 3 d later. We observed two distinct populations of neutrophils within the draining lymph nodes at 16 h postinjection: $CD45.2^+$ resident neutrophils and $CD45.1^+$ donor neutrophils. The proportion of neutrophils did not change significantly despite FOXO1 knockdown, suggesting that FOXO1

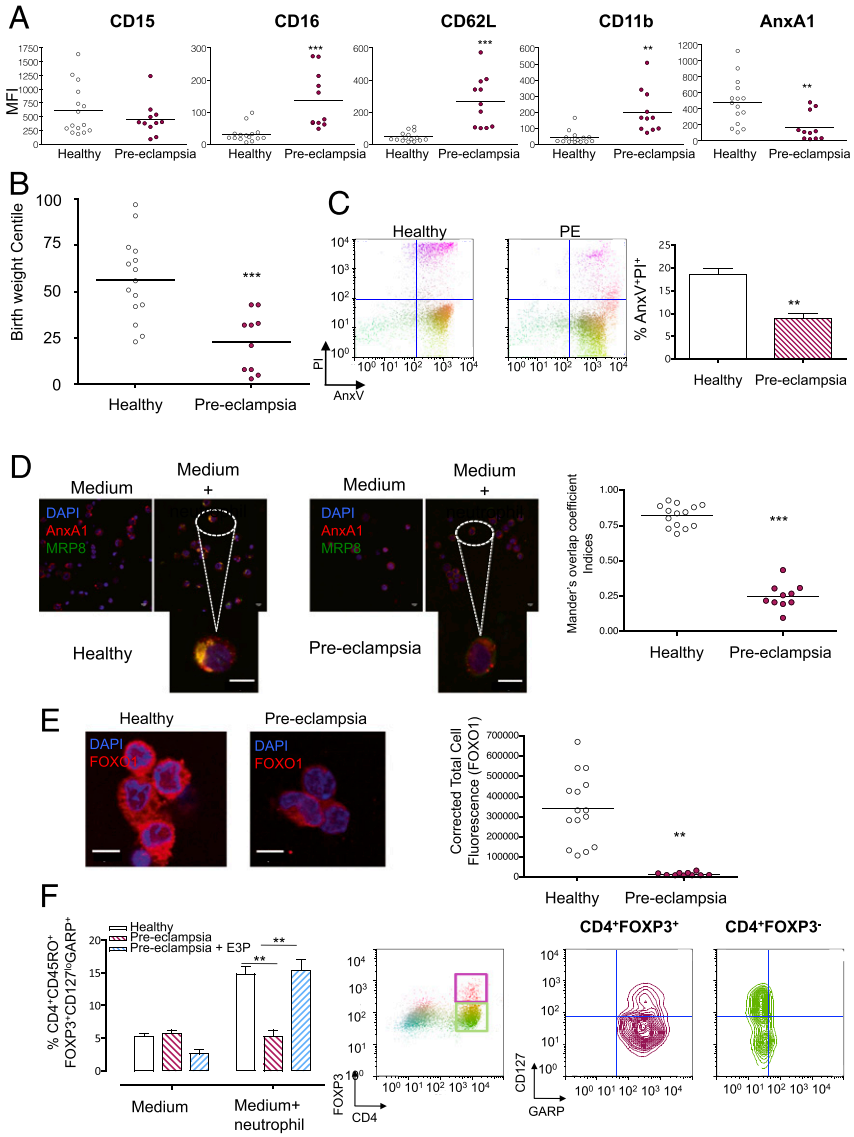

Fig. 6. Neutrophils from preeclamptic pregnancies fail to induce niT cells. (*A*) Peripheral blood neutrophils from patients with preeclampsia were stained for CD15, CD16, CD62L, CD11b, and AnxA1 to establish their phenotype compared with cells from age- and gestation-matched healthy pregnant women (*SI Appendix*, Table S1). Data are mean ± SEM of 15 healthy and 10 preeclamptic samples. ***$P < 0.001$; **$P < 0.01$ using Student's *t* test for each marker. (*B*) Comparison of gestation-adjusted birth weight percentiles for babies born from normotensive mothers ($n = 15$; mean, 52nd percentile; range, 44th–68th percentile) and preeclamptic mothers ($n = 10$; mean, 22nd percentile; range, 11th–35th percentile). (*C*) Extent of apoptosis of peripheral blood neutrophils from healthy and preeclamptic samples measured by annexin-V and PI labeling. Data are mean ± SEM of 15 healthy and 10 preeclamptic samples. **$P < 0.01$ compared with healthy pregnancy. (*D*) T cells from healthy and preeclamptic pregnancies were cocultured for 5 d with autologous neutrophils (no additional hormones added) and subsequently stained for DAPI (blue), AnxA1 (red), and MRP8 (green). The amount of neutrophil protein transferred to T cells was analyzed by confocal microscopy, and colocalization of AnxA1 and MRP8 was analyzed using Mander's overlap coefficient indices. Data are mean ± SEM of 15 healthy and 10 preeclamptic samples.***$P < 0.001$. (Scale bar: 7 μm.) (*E*) Neutrophils from healthy and preeclamptic donors were cocultured with autologous T cells as described in *B*. T cells were then stained for DAPI (blue) and FOXO1 (red) and analyzed by confocal microscopy to establish the efficiency of FOXO1 transfer to the T cells, as measured by CTCF. Data are mean ± SEM of 15 healthy and 10 preeclamptic samples. ***$P < 0.001$. (Scale bar: 7 μm.) (*F*) Neutrophils from healthy and preeclamptic patients were cocultured with autologous T cells at a 1:1 ratio for 5 d in the presence of 2 μg/mL soluble anti-CD3 and anti-CD28 antibodies. T cells were stained for niTreg markers as in Fig. 2. In addition, neutrophils from preeclamptic patients were treated with exogenous E3P and then cocultured with T cells (blue lined bar). Data are mean ± SEM of three experiments with 15 healthy and 10 preeclamptic samples; **$P < 0.01$.

is not required for migration of neutrophils to the draining lymph nodes; however, we did not find any CD45.1$^+$ donor neutrophils in nonpregnant controls (Fig. 7*B*, contour plots). At 3 d postinjection (day 12 of pregnancy), we failed to see any live CD45.1$^+$ donor neutrophils (Fig. 7*B*, dot plots). This finding was expected, given that neutrophils need to undergo apoptosis to induce Tregs in this system. Interestingly, despite the low number of CD45.1 Ly6G$^+$ neutrophils identified in the draining lymph nodes, we still observed no difference in total number of Ly6G$^+$ neutrophils in the draining lymph nodes (Fig. 7*A*, *Left*), suggesting that migration of endogenous neutrophils to the draining is not affected. This could be related to the IL-17 made by T cells in the draining lymph nodes (*SI Appendix*, Fig. S13*C*) following reconstitution of nonspecific transduced neutrophils and, to a lesser extent, FOXO1 knockdown neutrophils, because this cytokine has been shown to promote neutrophil migration into draining lymph nodes (47).

Although we did not detect CD45.1 neutrophils in the drainage, we did detect a CD45.1$^+$ signal within the CD4$^+$FOXP3$^+$

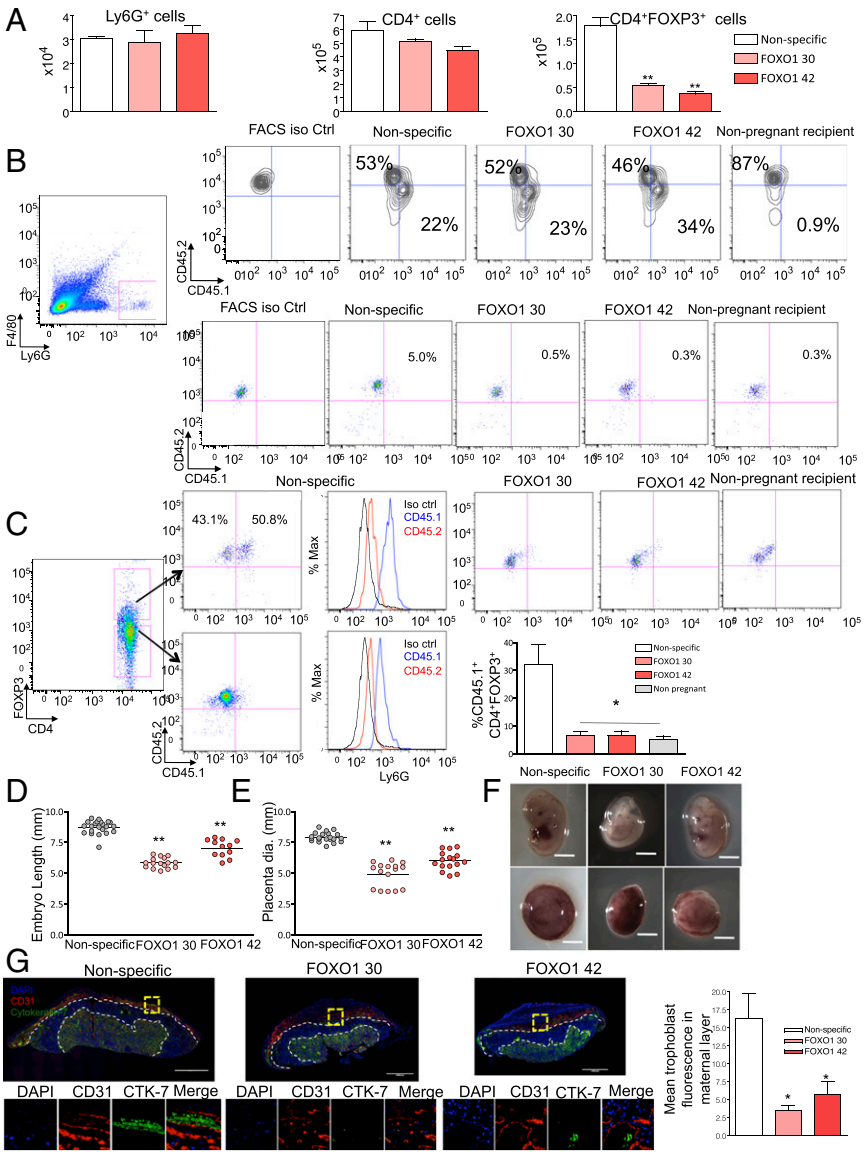

Fig. 7. Knockdown of FOXO1 in neutrophils leads to abnormal pregnancy. (*A*) Balb/C males were mated with C57 BL/6 females and neutrophil-depleted as described in Fig. 3. Bone marrow progenitors were transduced to knock down FOXO1, followed by culture with 100 ng/mL GCSF for neutrophil differentiation. Then 3×10^6 transduced neutrophils were injected i.v into the tail vein at days 6 and 9 of pregnancy, and mothers were killed at day 12. Absolute numbers of Ly6G$^+$ neutrophils, CD4$^+$ T cells, and CD4$^+$FOXP3$^+$ Tregs in the uterine-draining lymph nodes were counted. $n = 3$ mice per group from three distinct timed matings. $P < 0.01$. (*B*) Bone marrow from CD45.1$^+$ donor mice was transduced with nonspecific neutrophils reconstituted as described above, and the presence of donor CD45.1 Ly6G$^+$ (clone Gr-1) F4/80$^-$ neutrophils was analyzed by flow cytometry after 16 h (contour plots) and after 72 h (day 12) postinjection. (*C*) The presence of transferred CD45.1 donor neutrophil material to the CD4$^+$FOXP3$^+$ population was also analyzed by flow cytometry, and its origin was confirmed by Ly6G staining, compared with CD45.2$^+$ cells. $n = 3$–4 mice per group from three distinct timed matings. (*D* and *E*) Embryo size (*D*) and placenta diameter (*E*) were measured as described in Fig. 3. $n = 22$ embryos and placentas from nonspecific, $n = 16$ from FOXO1 30, and $n = 12$ from FOXO1 42 from three distinct pregnancies per group. $^{**}P < 0.01$ compared with nonspecific. (*F*) Representative images of embryo and placenta of all three groups. (Scale bar: 2.5 mm.) (*G*) Representative immunofluorescence images of placentas for CD31 (red), trophoblasts (cytokeratin-7; green), and cell nuclei with DAPI (blue). Dotted lines indicate placental layers as described in *E*. The yellow dotted square indicates the zoomed-in section of the Db layer to more closely examine trophoblast invasion. Invasion of trophoblasts into the maternal layer was quantified using ImageJ software. $n = 3$ placentas from each group. $^*P < 0.05$ compared with nonspecific. In *A* and *F*, data are mean ± SEM; in *B* and *C*, the line indicates the mean.

T cell population (Fig. 7*C*). Importantly, this effect was observed only when mice were injected with CD45.1 neutrophils transduced with nonspecific virus, and not with FOXO1-depleted CD45.1 neutrophils. The CD45.1$^+$ signal within the Treg population was of neutrophil origin, because it was accompanied by Ly6G staining (which was not seen on CD45.2$^+$ cells; Fig. 7*C* for histograms and *SI Appendix*, Fig. 13*F* for singlet gating). Moreover, this phenomenon was pregnancy-specific, because injection of CD45.1 neutrophils into nonpregnant females did not yield a CD45.1 signal in the Treg population recovered from the draining lymph nodes, and was not seen in the nondraining lymph nodes or spleens of pregnant mice (Fig. 7*C* and *SI Appendix*, Fig. 13*G*). Taken together, the foregoing data conclusively demonstrate that neutrophils are able to transfer their protein content directly to T cells in vivo to induce Tregs during pregnancy and is hindered in the absence of neutrophil-derived FOXO1.

Both embryo and placenta sizes from pregnant mice reconstituted with FOXO1-sufficient neutrophil were analogous to

those in mice that did not undergo neutrophil depletion (Fig. 7 *D–F*, *Left*), coupled with an organized placental structure and normal trophoblast invasion into the maternal layer (Fig. 7*G*, *Left* and *SI Appendix*, Fig. 13*H*). Pregnant mice reconstituted with FOXO1-knockdown neutrophils yielded significantly smaller embryos and placentas (Fig. 7 *D–F*, *Middle* and *Right*). The placental layers of these mice displayed a disorganized morphology with poor trophoblast invasion and were comparable to placentas following neutrophil depletion (Fig. 7*G*, *Middle* and *Right*). This difference in placental phenotype between total loss of neutrophil (depletion) and selective loss of neutrophil FOXO1 suggests that, in addition to niT-cell induction by FOXO1 transfer, maternal neutrophils might exert other direct effects (i.e., niT cell-independent) on placental development.

Discussion

The ability of neutrophils to impact adaptive immunity has recently emerged (10), although it has not been considered in the context of a regulatory/tolerant phenotype. Here we describe a role for a specific neutrophil functional phenotype—consequent to exposure to maternal and placental hormones—in the induction of a population of proangiogenic T cells with regulatory properties in pregnancy. The differentiation of niT cells is reliant on AnxA1-facilitated transfer of FOXO1 contained in neutrophil apoptotic bodies to T cells during activation; niT cells are required to maintain normal pregnancy outcomes as depletion of neutrophils during pregnancy leads to smaller embryo sizes and abnormal placentation in mice. Whereas the presence of activated neutrophils in the maternal circulation has been reported to be detrimental in pregnancy complications, including preeclampsia (22, 48), our observations suggest that a regulatory nonactivated proapoptotic neutrophil phenotype promoted by pregnancy hormones might be essential for normal placentation, including its vascular development. The cytokine milieu that results from niT-cell generation could favor the establishment of a proangiogeneic environment, with both IL-10 and IL-17 promoting vessel development (21, 49) and IL-17 promoting trophoblast invasion (27, 50), both key processes in spiral artery remodelling (26). In addition, and more specifically, we have shown that niT cells make IL-17–dependent VEGF, lending further support to their proangiogenic function within the placenta.

Analyses carried out in samples from women with preeclampsia provide pathological relevance to this mechanistic work, identifying defects in this physiological circuit that may be contributory to poor pregnancy outcomes of preeclampsia. During healthy pregnancy, the maternal immune system adapts to allow survival of a partially histoincompatible fetus, and failure of this adaptation contributes to poor placental invasion that predates the clinical onset of preeclampsia. Studies have suggested that impaired expansion of inducible Treg (iTreg) cells (40, 51), particularly in the decidua, might represent a pathogenic defect in preeclampsia. A key feature of the niT-cell population that we describe is its ability to produce IL-17. Whereas there is general agreement that in healthy pregnancy, there is a preferential differentiation of iTreg cells over Th17 cells systemically, the functional significance of Th17 cells in the decidua is controversial. An increased decidual Th17 cell percentage in women with unexplained recurrent miscarriages compared with those with healthy pregnancies has been described previously (52); however, another study reported higher percentages of Th17 cells in the decidua in healthy pregnancies (27). Our observations suggest that IL-17–producing niT cells rather than "conventional" Th17 effectors in the placenta might in fact be required to support vascular development in healthy pregnancy, given that IL-17 is known to promote angiogenesis and tissue growth (21).

Neutrophil-derived FOXO1 plays an important role in niT-cell induction and the maintenance of embryo size and normal placentation. FOXO1 is a known important transcription factor that promotes FOXP3 expression in T cells; however, FOXO1 has broader implications in placentation and indeed is necessary for normal placental development, as indicated by the abnormalities observed in FOXO1-null mice (38). Similarly, the role of Tregs in pregnancy might not be confined to regulating immune responses; broader effects of Tregs on the maternal vasculature have been reported, with downstream relevance in controlling hypertension and other cardiovascular events (53). Moreover, Tregs can modulate cell surface proteins on endothelial cells, leading to an anti-inflammatory endothelial cell phenotype (54). Thus, it is tempting to speculate that failure to promote appropriate niT-cell generation might prevent the establishment of a proangiogenic environment within the placenta. Further studies are warranted to test this hypothesis.

The induction of niT cells by neutrophils likely occurs physiologically in the uterine-draining lymph nodes during the presentation of paternal antigens to circulating allospecific T cells, as reported previously (55). In this context, we have shown that neutrophils can access lymph nodes where they exchange cellular material with T cells. In our system, neutrophil protein transfer occurs selectively in the uterine-draining lymph nodes, the only likely site of active immunity during pregnancy in otherwise healthy mice, suggesting that T-cell activation is also required for the transfer of neutrophil material—as we have shown in our in vitro studies—and that TCR triggering likely induces a permissive status for the "acceptance" of neutrophilic proteins into T cells. The molecular features of this permissive status remain to be established.

Importantly, as the induction of this neutrophil phenotype occurs in the absence of other signals (e.g., inflammatory) as a consequence of exposure to circulating hormones, it is conceivable that this functional status might arise in neutrophils systemically during pregnancy and might affect ongoing T-cell activation in sites other than the fetal-maternal unit—hence the well described remission of antigenically unrelated autoimmune responses during pregnancy (56).

In conclusion, we report a cell-to-cell cross-talk whereby E3P programs neutrophils to induce proangiogenic T cells, thus enabling sustainable placenta/fetus interface development. We predict that neutrophil regulatory properties in several facets of the adaptive immune response, tolerance in particular, can be further identified to decipher the way in which innate and adaptive immune systems cooperate, providing both opportunities for defining new pathogenic mechanisms and novel modes for therapeutic interventions.

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